REMARKS

Claims

Claims 1–2, and 5–12 are currently under examination with claims 3, 4, and 13–18 previously withdrawn due to restriction/election.

Claim amendments

The claims are amended to recite language in accordance with conventional US practice. It is courteously submitted that the claim amendments do not raise new matter.

Specification

A separate sheet containing the ABSTRACT of the instant specification, as originally filed (International Application PCT/EP2003/009315), is enclosed herewith. See, USPTO records of February 1, 2005 on the Patent Application Information Retrieval (PAIR) database. Inasmuch as the enclosed ABSTRACT fully complies with the guidelines stated under M.P.E.P. §608.01(b), it is submitted that the outstanding objection of the abstract should be duly withdrawn.

The specification has been amended to include "Brief Description of the Drawings." Support for the amended can be found, for example, the paragraphs bridging page 9, line 18 to page 10, line 6 (Figure 1); page 17, lines 30-31(Figure 2); page 18, lines 26-27(Figure 3); and page 21, lines 3-4 (Figure 4) of the specification, as originally filed. No new matter is added.

Withdrawal of the objection is respectfully requested.

Rejection under 35 U.S.C. §103(a)

The contention that Bazin (Spectrochimica Acta, **vol. 57**, pages: 2197–2211, **2001**) in view Nicholson (US 4,859,581) renders obvious the subject matter claimed in claims 1, 2, and 5–12 is respectfully traversed.

The assay method disclosed in Bazin relates to labeling of a peptide substrate "on both ends" and "at known positions" of the peptide substrate. Forexample, all illustrative examples provided in the cited reference are strictly drawn to the detection of biological material or substrates of a known chemical structure, and that are specifically engineered so the donor and acceptor fluorophores will both bind at known positions on the substrate. It is further described that this method of engineering of substrate molecules achieves close proximity, which will result in a fluorescent signal. In this respect, the Examiner is courteously requested to refer to the following paragraphs of the cited reference, wherein it is expressly taught that:

> p. 2205, 1st column, last paragraph: "Cummings and coworkers built up an HIV protease format by tagging both ends of a peptide respectively with biotin and a sequence containing phosphotyrosine used here as a tag."

p. 2205, 2nd column, 1st paragraph: "...Takemoto et al performed a Caspase 3 indirect assay. A7 amino-acid peptide was labeled at both ends with biotin and DNP groups"

p. 2206, 3.2.2 "A peptide containing a sequence recognized by a protease was labeled on one side with XL665 and on the other side with biotin. (Emphasis added)

Therefore, Bazin et al., as far as proteases are concerned, teach that the enzyme substrate must be labeled – either directly or indirectly – "on both ends" (i.e., both sides) of the cleavage site.

A skilled artisan understands that a peptide is made of a known combination of the 20 natural amino-acids; tools for peptide analysis are generally well-described in the art, as well as techniques relating to chemical synthesis of such peptide molecules. Moreover, proteases cleave peptide substrates at known locations and sequences. When developing a FRET-based protease assay, the skilled artisan understands that there is little left to chance: the cleavage site of the peptide and the coupling-site of the donor and acceptor fluorophore are to be precisely known. However, the substrates of the instant invention, which can be used to assay the claimed endoglycosidase activity, for example, heparan sulfate proteoglycan, ECM-associated heparan sulfate, heparin, or heparan sulfate, containing from 1 to 30 units of glycosidic monomers, are structurally distinct from the substrates claimed by Bazin et al.

Furthermore, labeling such substrate molecules (for e.g., heparan sulfate substrate) at precisely known locations is a matter of such technical complexity in that the aforementioned skilled artisan, relying on the well-established knowledge and/or tools of peptide chemistry, cannot comprehend how to do so routinely. This is especially applicable in the development of a FRET-based glycosidase assay, wherein the substrate is not a well-characterized peptide, but a mix of heparan sulfates (HS) or HS proteoglycans (HSPG) of different and unknown sequences and structures. This results in the fact that neither the cleavage site nor the exact position of the donor and acceptor fluorophore can be wholly presumed in such substrate molecules.

Heparan sulfate (HS) is a linear polysaccharide consisting of a chain of disaccharide units of N-acetyl-D glucosamine that is linked to D-glucuronic acid. The disaccharided repeat units *can be* (but are not necessarily) modified to include N- and O- sulfation (6-O and 3-O sulfation of the glucosamine, and 2-O sulfation of the uronic acid) and epimerization of beta-D-glucuronic acid to alpha-L-iduronic acid. Together, the five different modifications for disaccharides give rise to 32 combinations. Thus, the complexity of heparan sulfate is even greater than that of proteins, which are made up of 20 aminoacids. With these 32 building blocks, a heparan sulfate octasaccharide could have over a million possible sequences.

According to Codée (Drug Discovery Today: Technologies, vol. 1, pp. 317-326, 2004), "the highly complex structure of heparan sulfate presents a formidable synthetic challenge and the incorporation of the full array of variations in oligosaccharides of significant length is a daunting task." See, the INTRODUCTION section of of Codée et al, 2004, a copy of which is enclosed herewith for the Examiner's review. Moreover, the enclosed reference teaches that "it is virtually impossible to obtain specific heparan sulfate from natural sources owing to their microheterogeneity." See, for example, the disclosure contained in col. 2, p. 317 of Codée et al.

Consequently, the teaching of Bazin et al., which is drawn to FRET-based assay of protease activity, is not applicable to a FRET-based assay of an endoglycosidase activity. Since the skilled artisan appreciates the nature of the substrate compounds involved in the assay of such endoglycosidases, for example, heparan sulfate substrate and the like, and further has no knowledge on labeling of this substrate "on both ends" (i.e., on both sides of a cleavage site), the simplicity of Bazin's methods and substrates cannot be used in the claimed technique of endoglycosidase assay.

The Office Action then contends that the deficiencies of Bazin are rectified by the secondary reference of Nicholson (US 4,859,581), which is drawn to a chromatographic method of detection. The fundamental differences between the claimed method(s) and Nicholson's technique have been previously established. For example, Nicholson's disclosure requires "separation of the reaction products from the substrates based on their size." In contrast, Applicants' claimed invention does not necessitate size-resolution. Moreover, the secondary reference of Nicholson merely discloses the use of 'fluorescein-labeled HS for a rapid analysis of degradation fragments on HPLC equipped with a flow fluorescence detector." These are not donor-acceptor compounds which are in close proximity to one another. The cited reference does not describe a method for assaying for endoglycosidase activity by utilizing a substrate which is directly or indirectly labeled with a first donor compound and with a second acceptor compound, and in that the amount of intact substrate is determined by measuring a signal emitted by the acceptor compound, this signal resulting from a transfer, via a close proximity effect, between the donor and the acceptor compounds.

It is therefore courteously submitted that Bazin fails to provide any hint or suggestion to one of ordinary skill that the peptide-peptidase (substrate-enzyme) system could be applied in a complex and the challenging assay technique such as one described by the instantinvention. Absent such guidance, one of ordinary skill in the art would not be motivated to reformulate the

cited references to arrive at the instant invention. Moreover, even if were to combine the teachings imparted by Bazin and/or Nicholson, the cited references, even at their broadest disclosure fail to render obvious the subject matter of the instant invention. There is no mention of employing a FRET-based assay technique for the measurement of endoglycosidase activity. Therefore, a combination of Nicholson and Bazin cannot render obvious the subject matter of the instant invention.

Furthermore, Applicants submit that a skilled artisan would also appreciate differences between heparanase assay as compared to the heparitinase assay, as recited in the instant claims. See, for example, Applicants' claim 5. For example, additional technical challenges appear with heparanase enzymes: Heparanase cleaves HS into large fragments, as compared with other HS hydrolase such as heparitinase. Consequently, there are less cleavage sites for this enzyme, which increases the technical difficulty hurdle of labeling the substrate "on both sides" of a cleavage site. The added functional complexity of the claimed enzymes, and the technical complexity of assaying for such, is not possible with the generic chromatographic technique disclosed by Nicholson.

In view of the above remarks, it is courteously submitted that the outstanding rejection be withdrawn. If there are any remaining issues which could be expedited by a telephone conference, the Examiner is courteously invited to telephone counsel at the number indicated below.

No fees are believed to be due with this response; however, the Commissioner is hereby authorized to charge any fees associated with this response to Deposit Account No.13-3402.

Respectfully submitted,

Anthony J. Zelano, Reg. No. 27,969
Attorney for Applicant(s)

MILLEN, WHITE, ZELANO
& BRANIGAN, P.C.
Arlington Courthouse Plaza 1, Suite 1400
2200 Clarendon Boulevard
Arlington, Virginia 22201
Telephone: (703) 243-6333
Facsimile: (703) 243-6410

Attorney Docket No.: **LOM-0047**

Date: **June 21, 2007**

Encl: Codée et al., *Drug Discovery Today: Technologies*, vol. 1, pp. 317-326, 2004

ELSEVIER

DRUG DISCOVERY TODAY TECHNOLOGIES

Drug Discovery Today: Technologies

Vol. 1, No. 3 2004

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Lead optimization

The synthesis of well-defined heparin and heparan sulfate fragments

Jeroen D.C. Codée[1,‡], Herman S. Overkleeft[1], Gijsbert A. van der Marel[1], Constant A.A. van Boeckel[2,*]

[1]Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands (http://www.leidenuniv.nl/)
[2]Medicinal Chemistry, Organon N.V., P.O. Box 20, 5340 BH Oss, The Netherlands (http://www.organon.nl/)

Heparin and heparan sulfate are key players in a plethora of physiological processes. Organic synthesis is the method of choice for the production of these oligosaccharides and their derivatives and analogues. The highly complex structure of these polysaccharides presents a formidable synthetic challenge and the incorporation of the full array of variations in oligosaccharides of significant length is a daunting task. This review records the development of strategies to access these exciting biomolecules.

Section Editor:
Constant A.A. van Boeckel – Medicinal Chemistry, Organon N.V., Oss, The Netherlands

Difficulties in the isolation of well-defined heparin and heparan sulfate fragments have led to the use of organic synthesis for the production of such oligosaccharides. Here, Codée *et al.* review several synthetic methodologies and target-oriented and modular approaches that are used for the development of heparin and heparan sulfate fragments. van Boeckel was involved in a long-standing joint venture between Organon and Sanofi, generating novel synthetic heparin antithrombotics; as an adjunct professor he collaborated for many years with the other authors, who are experts in carbohydrate chemistry at Leiden University. The group is currently headed by Professor Hermen Overkleeft. Jeroen Codée has recently completed his PhD; his thesis describes new synthetic methodologies of glycosaminoglycans in solution and on solid support.

Introduction

Heparin and heparan sulfate (H/HS, Fig. 1a) are negatively charged, unbranched carbohydrate polymers and present the most complex members of the glycosaminoglycan (GAG) superfamily, which further includes dermatan sulfate, chondroitin sulphate, keratan sulfate and hyaluronic acid. H/HS are built up from alternating glucosamine and uronic acid (D-glucuronic or L-iduronic) residues, which can be highly functionalised: *O*-sulfation can occur on the 2-position of the uronic acids and the 3- and 6-position of the glucosamine while the amino functions might be sulfated, acylated or unsubstituted. H/HS-polysaccharides interact with over a hundred proteins and have a crucial position in the regulation of various physiological processes [1]. To understand the mode of action of H/HS at a molecular level it is of prime importance to have access to pure, well-defined H/HS-fragments. Because it is virtually impossible to obtain specific H/HS-structures from natural sources (owing to their microheterogeneity), organic synthesis has become the method of choice for the production of GAG-oligosaccharides as well as their derivatives and analogues [2–5]. This review focuses on the synthesis of H and HS, because (1) they are the most complex members of the GAG superfamily and (2) the most important technological and methodological advancements in the field of GAG-synthesis have come from the research on H/HS-assembly.

Synthetic methodologies

The synthesis of H/HS-polysaccharides presents several challenges, imposed by the complex structure of the target mole-

*Corresponding author: (C.A.A. van Boeckel)
stan.vanboeckel@organon.com
‡ Present address: Laboratorium für Organische Chemie, ETH Hönggerberg, Wolfgang-Pauli-Strasse 10, CH-8093 Zürich, Switzerland (URL: http://www.ethz.ch/).

 DOI: 10.1016/j.ddtec.2004.11.017

Glossary

Modular approach: a synthetic strategy that employs closely related building blocks (modules). The similarity of the building blocks should allow for standardized condensation reactions and provide flexibility and variety in use.

Non-glycosamino glycans: synthetic glycosaminoglycans which bear *O*-methyl functions instead of free hydroxyls and *O*-sulfate groups in place of *N*-sulfates.

cules [2–5]. First, L-idose and L-iduronic acid are not readily available from commercial or natural sources, and therefore efficient synthetic routes to access sufficient amounts of these monosaccharides are required. The next challenge is the development of a suitable protecting-group strategy to allow the implementation of the high degree of functionalization of the H/HS fragments (Fig. 1b). In the vast majority of H/HS syntheses, the following sequence of events completes the construction of the H/HS-fragment: (1) selective deprotection of the hydroxyls to be sulfated; (2) sulfation of these hydroxyls; (3) deprotection of the remaining alcohol and amino functions; and (4) selective sulfation of the free amines. Generally to accommodate these manipulations in the synthesis, the free and sulfated alcohol functions are masked as benzyl ethers and ester groups (e.g. acetyl, benzoyl and pivaloyl), respectively. The amino functions are often taken through the synthesis as the corresponding azides. Next to these protecting groups an additional set of orthogonal groups (e.g. silyl ethers, levulinoyl esters, 9-fluorenyl methoxy carbonyl carbonates, *p*-methoxybenzyl and allyl ethers) is required to temporarily block the reducing and/or non-reducing termini. The last challenge, imposed by the H/HS structure, is the stereoselective and efficient formation of the interglycosidic bonds in the carbohydrate backbone. For the stereoselective formation of the α-glucosamine linkages, a non-participating group at C-2 (e.g. azide) of the glucosamine building block is required, whereas the C-2 of the uronic acid motives should be equipped with a participating acyl function. The presence of the carboxylate functions is of profound influence on the efficiency of the glycosylation reactions, because the electron-withdrawing carboxyl group at C-5 not only significantly reduces the nucleophilicity of the proximal OH-4 group but also lowers the glycosyl-donor reactivity of the uronic acid building blocks compared with their non-oxidised counterparts.

Target-oriented approaches

The first efforts towards the assembly of H/HS fragments were directed towards the synthesis of the anticoagulant heparin pentasaccharide (**1**), also known as the fragment DEFGH (Fig. 1b, structure **1**) [4,5]. This unique pentasaccharide sequence binds antithrombin III (AT III), a crucial inhibitor for the blood coagulation factors IIa (thrombin) and Xa (Fig. 1c,d). Whereas the pentasaccharide fragment is sufficient for inhibition of factor Xa, the inhibition of thrombin requires a longer heparin chain (>16 monosaccharides). In the latter case, the heparin chain functions to bind both AT III and thrombin in a ternary complex thereby guiding the protease (thrombin) towards the reactive center loop of its inhibitor (AT III) (Fig. 1d). Epic total syntheses of the highly functionalized oligosaccharide **1** were reported in the mid-1980s by the groups of Sinaÿ [6] and van Boeckel [7]. Ever since, a vast array of analogues has been synthesised to establish the SAR of the pentasaccharide domain [4,5]. One of the first analogues to be synthesised was pentamer **2** (Fig. 1b), the α-*O*-methyl glucoside analogue of **1** [8], which was recently launched as a novel antithrombotic drug by the pharmaceutical companies of Organon (http://www.organon.com/) and Sanofi (http://www.sanofi-aventis.com/) under the name Arixtra® (Fondaparinux). Capping the anomeric center of unit H with a methyl group simplifies the synthesis and purification of the saccharide significantly because the reactive aldehyde group at the reducing end is persistently protected. The initial syntheses of pentasaccharides **1** and **2** were based on the state-of-the-art oligosaccharide synthesis at the time and used glycosyl bromide and orthoester building blocks (**6–10**) in combination with the protecting-group strategy outlined above and required over 60 steps (retrosynthetically depicted in Fig. 1b). In the course of the development of *Arixtra*®, the synthesis of **2** has improved using building blocks **11** and **12**, as depicted in Fig. 1b, and much chemistry has been adjusted to facilitate a factory process. Following a target-oriented strategy designed for these pentasaccharides, a collection of analogues has been constructed including decarboxylated [4], phosphorylated [9], epimeric [4], flexible [4], desulfated [10] and supersulfated [4] analogues. The biological activities of these analogues point to the structure activity relationships depicted in Fig. 1b. Furthermore, using tailor-made conformationally restricted pentasaccharides, it was established that the L-iduronic acid moiety (a rather flexible motive in the DEFGH-sequence) has to adopt the $^{2}S_{0}$-conformation to exert its biological activity [11].

In the pentasaccharide field, an important synthetic step forward has been the development of the "NON-GLYCOSAMINO" GLYCAN (see Glossary) heparin oligosaccharides (e.g. **3** and **4**, Fig. 1b), which bear *O*-methyl functions instead of free hydroxyls and *O*-sulfate groups in place of *N*-sulfates (technology developed by Organon N.V. and Sanofi-Aventis) [12]. These modifications dramatically simplify the assembly of H/HS-like fragments because (1) the deprotection and sulfation sequence at the end of the oligosaccharide is more straightforward and requires less steps; (2) glucose instead of glucosamine building blocks can be used; and (3) a larger choice of protecting groups is available. In addition, the last two issues

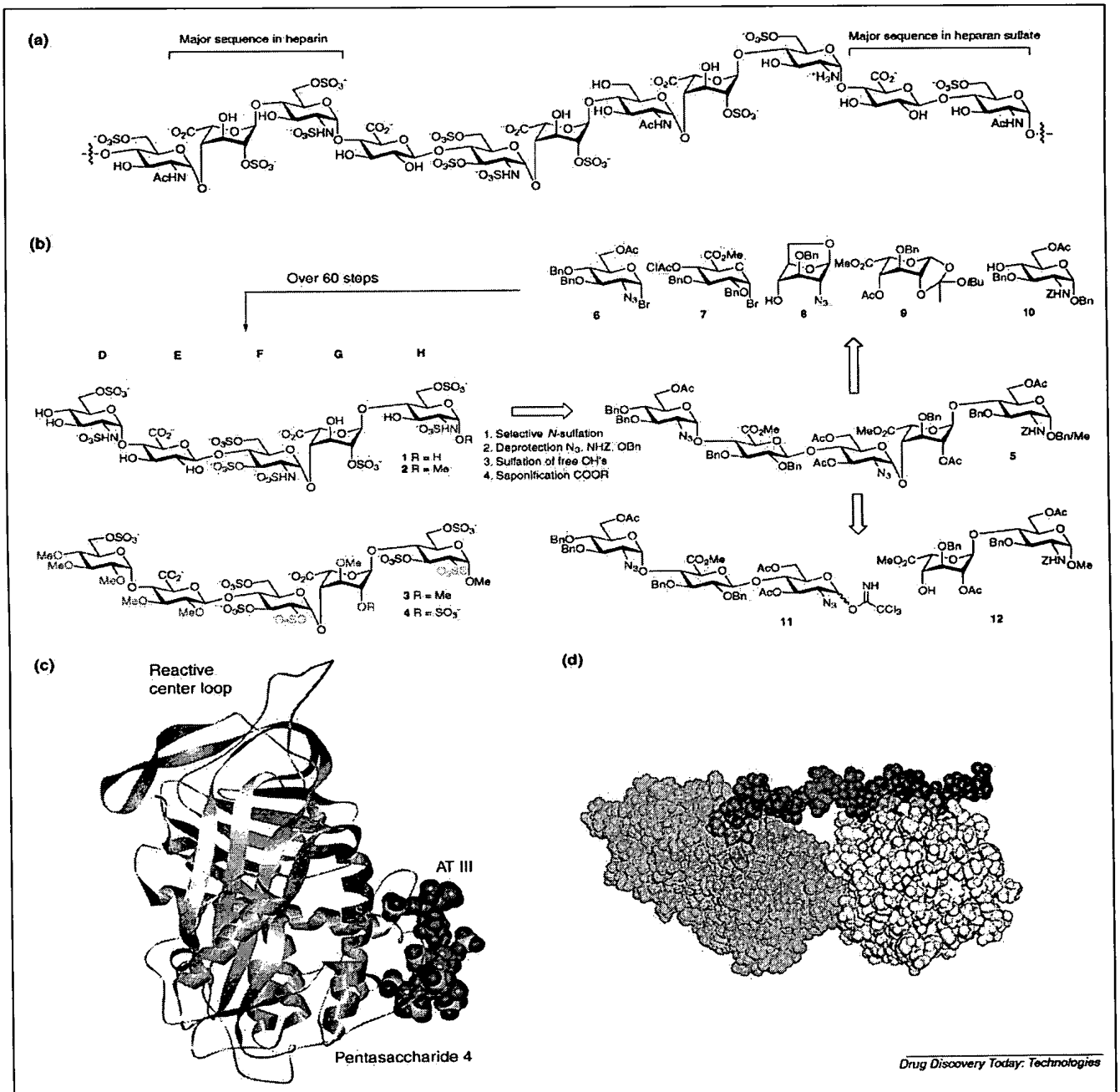


Figure 1. The anticoagulant heparin pentasaccharide story. (a) Heparin/heparan sulfate (H/HS) polysaccharide. H and HS differ in substitution pattern (H contains on average 2.7 and HS only 1 sulfate group) and glucuronic to iduronic acid ratio (for H: 1:9, for HS: 6:4). (b) Synthetic strategy used for the synthesis of the H-pentasaccharides **1** and **2**. Groups in **1** and **2** highlighted in red are essential for antithrombin III (AT-III) activation, whereas groups depicted in blue contribute to biological activity. The non-GAG analogues (**3** and **4**) bear *O*-methyl groups (in green) and *O*-sulfate groups (orange) instead of free hydroxyls and *N*-sulfates, respectively. (c) Crystal structure of AT-III in complex with non-GAG pentasaccharide **4** [33]. (d) Molecular model of the ternary AT-III–heparin–thrombin complex, which shows that heparin forms a bridge between the two proteins. Six to eight monosaccharide residues do not interact with the proteins [34].

have a major impact on the reactivity of the donor and acceptor building blocks in the glycosylation reactions *en route* the target oligosaccharide. Besides these synthetic advantages (the total synthesis of **3** comprises approximately 25 steps), the non-GAGs have also provided more potent pentasaccharide analogues. Pentamer **3** (Idraparinux) has an anti-Xa activity of 1600 U/mg as opposed to the reactivity of 700 U/mg of the "natural" pentamer **2**. Idraparinux is in late clinical trials and because of its high affinity to AT III has a very long half-life in circulation which allows once a week administration only [13].

The more straightforward access to non-GAGs compared with GAGs is nicely illustrated by the synthesis of the impressive polysaccharides **15** [14] and **20** [15] (Fig. 2a). Eicosamer **15** was synthesized to study the interaction with both AT III and thrombin (factor IIa) via a ternary complex. Therefore, the polysaccharide had to contain both an AT III binding domain (ABD) and a thrombin-binding domain (TBD) separated by a spacer segment. At the time of synthesis it was unknown which terminus (the reducing or non-reducing) of the ABD had to be extended with the spacer moiety and the TBD. Therefore, oligosaccharides were synthesized which can be regarded as a continuum of ABDs. A hexasaccharide (**15**), which consisted of three identical iduronic acid–glucose disaccharide motives was selected as an ABD domain. This made it possible to synthesize the oligomers using one single dimer building block (i.e. **13**) in an iterative fashion. Employing this building block a hexa-, deca-, dodeca-, tetradeca-, hexadeca-, octadeca- and eicosasaccharide were assembled, of which the latter (**15**) proved to be the most potent thrombin inhibitor. Dreef-Tromp *et al.* [16] used similar imidate building blocks (e.g. **17**) in the first polymer supported synthesis of non-GAG heparin oligomers (Fig. 2b). The soluble monomethyl polyethylene glycol support (MPEG) was employed to synthesize a set of heparin oligosaccharides (**19**), which differed in length (up to 12-monomers) and sulfation pattern.

Another milestone was achieved by the impressive synthesis of the thrombin inhibiting heptadecasaccharide (**20**) (Fig. 2c) [15]. This non-GAG oligosaccharide is composed of an ABD that is connected at its non-reducing end to a TBD with a neutral oligosaccharide spacer. This extraordinary molecule was shown to be more potent than standard heparin and low-molecular mass heparin in models for both venous and arterial thrombosis. Furthermore, it showed a strong decrease of non-specific interactions with basic proteins (and hence less side effects, e.g. heparin-induced trombocytopenia) as a result of the incorporation of the neutral spacer motive.

Modular approaches

MODULAR APPROACHES (see Glossary) developed for the assembly of non-GAG heparin polysaccharides such as **15** and **19** have also been applied for the synthesis of the synthetically more challenging glycosaminoglycan H/HS. A modular strategy that is applicable to a variety of (dimer) building blocks, differing in their substituent pattern (acyl versus benzyl protecting groups, differentiated amino functionalities, iduronic versus glucuronic acid configuration) would open up the way to tackle the enormous diversity found in H/HS structure. Furthermore (automated) solid-phase protocols can then be envisaged to speed up the process of H/HS-oligosaccharide assembly. A variety of different approaches has been reported over the years, differing in the use of protecting groups in the building blocks, timing of the oxidation step to access the uronic acids, and glycosylation procedure [17–23]. However, no general strategy has evolved thus far that accommodates all of the characteristics displayed by the H/HS-oligosaccharides. The incorporation of glucuronic acid residues and the differentiation of the OH-2 of the uronic acids and the amino functionalities have only very scarcely been reported.

One of the first modular approaches, as reported by Tabeur *et al.* [17], was directed towards the assembly of oligomers corresponding to the regular sequence of heparin. The synthetic strategy, based on the use of dimer **21** (Fig. 2d), which bears close resemblance to the non-GAG building blocks **13** (Fig. 2a) hinges on the reliable stereoselective formation of the α-D-glucosazide-L-iduronic acid linkage.

The Martín-Lomas laboratory has focused on the elucidation of the activation mechanism of the fibroblast growth factors (FGFs) by glycosaminoglycans (Fig. 3a). Regular-sequence heparin oligosaccharides (e.g. hexamer **30**) were prepared following the strategy depicted in Fig. 3b [18]. In this approach, the pivotal building block consisted of a disaccharide having the glucosazide motive at its non-reducing end (**24** and **28**). The stereoselective formation of the interglycosidic iduronic acid–glucosamine bonds was guided by the 2-*O*-pivaloyl and 2-*O*-benzoyl groups, providing anchimeric assistance in the condensations. The OH-4′ function was liberated after each coupling event by cleavage of the benzylidene group, and ensuing regioselective benzoylation. Interestingly they also constructed fragments, having differentiated amino substituents, bearing either acetyl or sulfate groups (e.g. structure **35**, Fig. 3c) [22]. Incorporation of the *N*-acetyl glucosamine moieties was accomplished using building block **36**, a close analogue of dimer **24**. This building block proved to be a poor acceptor glycoside, thereby illustrating one of the remaining major challenges in heparin synthesis: the efficient incorporation of differently functionalised amino groups.

The Martín-Lomas laboratory extended their solution-phase chemistry to a solid-phase approach (see Fig. 3b) [25]. At first glance this might seem a trivial operation, but the use of building blocks of low reactivity seriously limits the possibilities for such a translation. Hence, a soluble solid

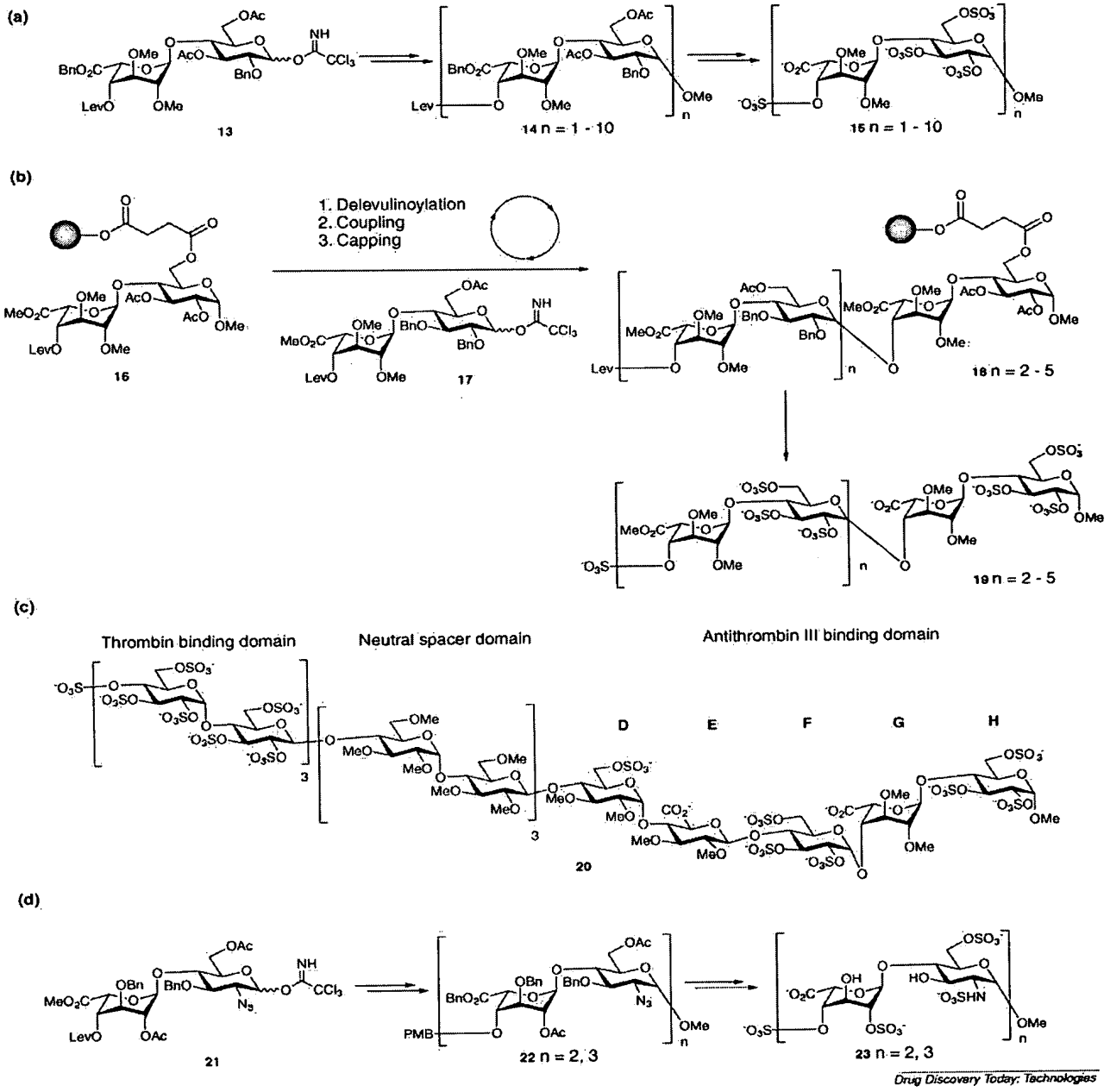
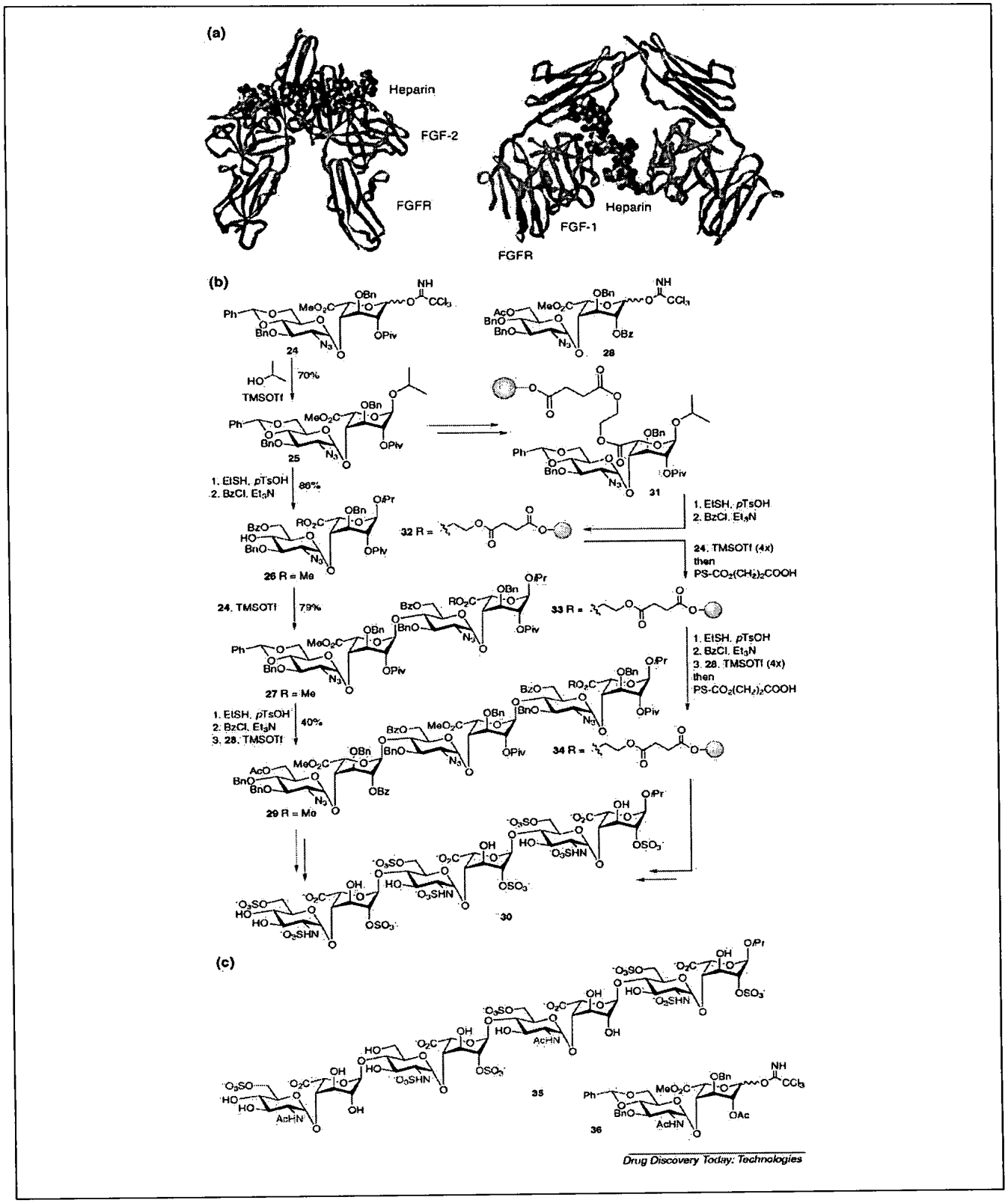


Figure 2. Synthetic approaches towards heparin-like oligosaccharides. (a) Modular approach towards an antithrombin active non-GAG heparin oligosacchairde. (b) First polymer support synthesis of non-GAG heparin oligosaccharides. (c) Synthetic thrombin inhibiting heparin-like heptadecasaccharide without side effects. (d) Modular approach towards the assembly of glycosamino glycan heparin oligosaccharides.

support (polyethylene glycol ω-monomethyl ether, MPEG) was chosen. A succinyl linker system, analogous to the linker used in the MPEG-based non-GAG synthesis of Dreef-Tromp *et al.* [16], was used for the attachment of the first disaccharide to the polymer. The crucial glycosylation events were executed using two equivalents of the imidate donor **24** or **28** in four repetitive cycles. Subsequently, the unreacted acceptor hydroxyls were scavenged with an acid functionalized solid support. This rather elaborate coupling cycle clearly indicates the low reactivity of the system. The partially protected


(a)
Heparin
FGF-2
FGFR
Heparin
FGF-1
FGFR
(b)
24
70%
TMSOTf
25
1. EtSH, pTsOH
2. BzCl, Et3N
86%
26 R = Me
24, TMSOTf
79%
27 R = Me
1. EtSH, pTsOH
2. BzCl, Et3N
3. 28, TMSOTf
40%
29 R = Me
30
28
31
32 R =
1. EtSH, pTsOH
2. BzCl, Et3N
24, TMSOTf (4x)
then
PS-CO2(CH2)2COOH
33 R =
1. EtSH, pTsOH
2. BzCl, Et3N
3. 28, TMSOTf (4x)
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PS-CO2(CH2)2COOH
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(c)
35
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Drug Discovery Today: Technologies

Table 1. Comparison summary table

	Technology 1	Technology 2	Technology 3
Name of specific type of technology	Solution-phase synthesis GAG	Solution-phase synthesis non-GAG	Polymer (MPEG) supported synthesis
Name of associated companies and company websites	Organon (http://www.organon.com/) Sanofi-Aventis (http://www.sanofi-aventis.com/)	Organon (http://www.organon.com/) Sanofi-Aventis (http://www.sanofi-aventis.com/)	Organon (http://www.organon.com/)
Pros	Natural targets	Efficient More protecting groups available More reactive synthons	Efficient Opens up ways for automation
Cons	Lengthy Elaborate protecting-group strategy Synthons of lower reactivity	Non-natural derivatives	Difficult glycosylation procedures Reduced reactivity of building blocks
References	[2–5]	[4,5,12]	[15,25]

hexasaccharide was obtained after basic cleavage/deprotection in 37% yield.

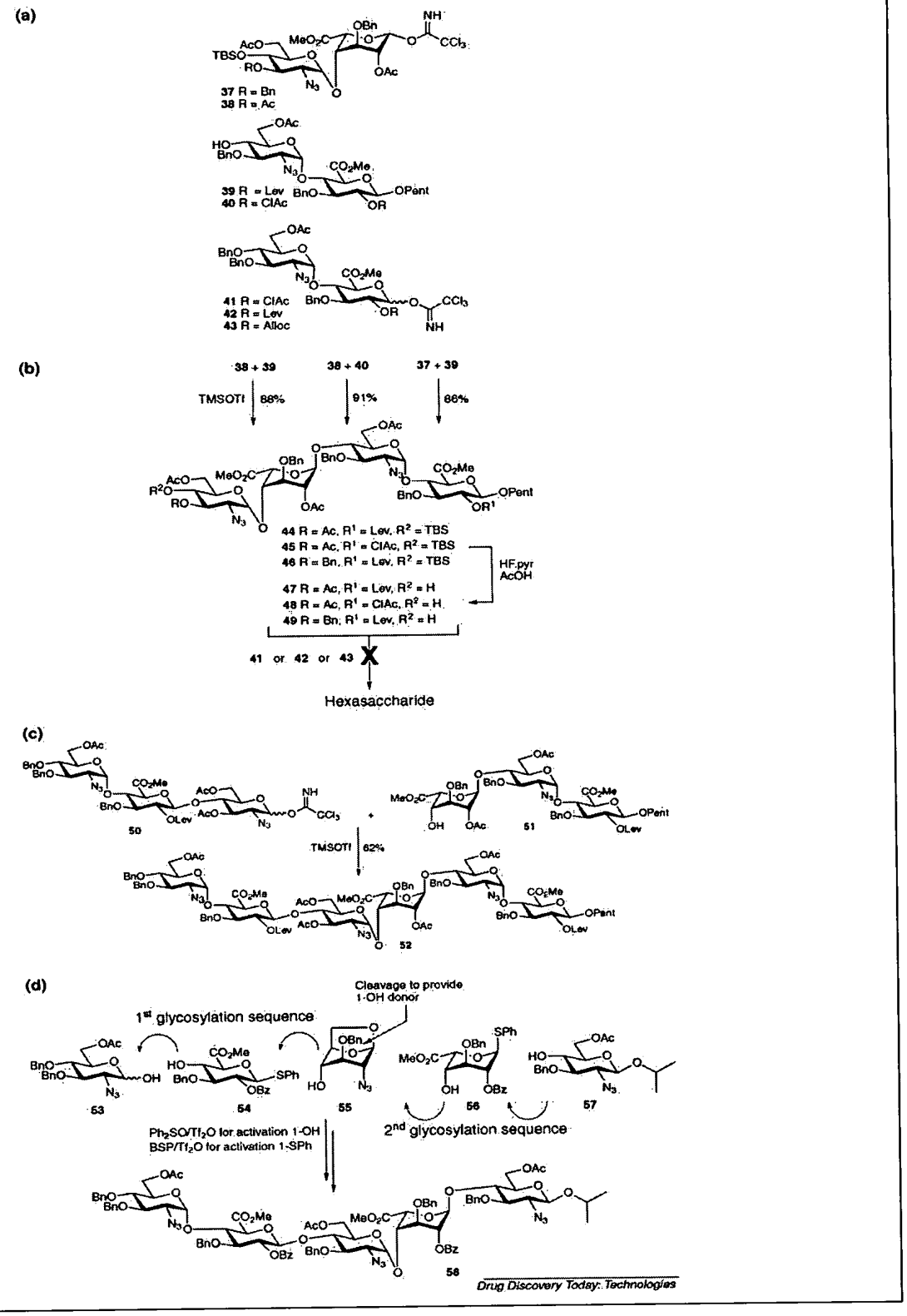
Seeberger and co-workers [19] set out to develop a modular approach using disaccharide building blocks having the uronic acid at the reducing end, as is depicted in Fig. 4a–c. They developed a novel method for the construction of the α-D-glucosazide-D-glucuronic acid linkage, in which the glucuronic acid acceptors were locked in the $^1C_4$-conformation [26]. Having constructed a collection of both glucosazido-iduronic acid (**37**, **38**, Fig. 4a) and glucosazido-glucuronic acid dimer building blocks (**39–43**, Fig. 4a), the modular journey towards larger heparin fragments was commenced. The tetrasaccharide stage was uneventfully reached providing a set of three highly diversified modules (**47–49**, Fig. 4b) for further elongation. In contrast to the success achieved in the above described syntheses of the Martín-Lomas laboratory [18,24,25], the Seeberger group [19] could not reach the hexasaccharide level using their envisaged [2 + 2 + 2] strategy. To get to the desired hexasaccharide fragment Seeberger and co-workers [19] rerouted their synthetic plan and continued with a [3 + 3] approach, relying on the stereoselective formation of the α-glucosazide–iduronic acid linkage (Fig. 4c). In the reported example, trisaccharide modules **50** and **51** were coupled to provide the highly functionalized protected hexasaccharide **52**, containing both glucuronic and iduronic acid residues. The unexpected failure of the [2 + 2 + 2] strategy can at the moment not be rationalized, but underscores the complex nature of GAG-synthesis [27].

A conceptually different modular approach was developed in the van der Marel laboratory. To accommodate for the high degree of diversity in the H/HS structure an assembly strategy was developed that is based on the use of monomeric building blocks in a sequential glycosylation strategy (Fig. 4d) [28]. This will allow the future incorporation of a diverse set of building blocks in a straightforward manner and avoids the use of far-advanced and therefore precious dimer motives. Another difference with the approaches reported above is the use of 1-hydroxyl and 1-thiodonors in a field in which imidate chemistry is almost exclusively encountered. Key building blocks in the strategy are the 1-thio glucuronic and iduronic acids, **54** and **56**, respectively. These building blocks were synthesized in a highly efficient manner, exploiting a regio- and chemoselective oxidation of partially protected 1-thio glucose and idose synthons [29]. Condensation of the inreactive thiodonors could be promoted by the use of recently developed powerful sulfonium activator systems [30,31]. A model pentasaccharide (**58**) was assembled in two consecutive condensation sequences from five monomeric building blocks (**53–57**) as is schematically depicted in Fig. 4d.

Conclusion

The strategies to access well-defined H/HS oligosaccharides each offer distinct (dis)advantages as summarized in Table 1. Comparing glycosaminoglycan H/HS synthesis to the assembly of non-GAG H/HS shows that the latter methodology offers great synthetic advantages: more temporary protecting groups are available, the building blocks used are more reactive, more robust (intermediate) structures can be obtained, and the deprotection/sulfation sequence at the end of the assembly is simplified. The evident setback of the non-GAG approach is that "non-natural" oligomers are generated. In the case of the anti-coagulant heparin pentasaccharide, this has proven to be no true disadvantage because it has been

Figure 3. Heparin and fibroblast growth factors (FGFs). (a) Structures of the FGF–FGFR–heparin complexes (reproduced with permission from [1]) [35]. The FGF is shown in green, the FGFR is depicted in orange. (b) Martin-Lomas's modular strategy of FGF-binding heparin oligosaccharides in solution and on a soluble polymer support (PS, polystyrene solid support). (c) Synthetic heparin oligosaccharide with differentiated amino functionalities, depicted in red and blue.


(a)
37 R = Bn
38 R = Ac
39 R = Lev
40 R = ClAc
41 R = ClAc
42 R = Lev
43 R = Alloc
(b)
38 + 39
38 + 40
37 + 39
TMSOTf
88%
91%
86%
44 R = Ac, R1 = Lev, R2 = TBS
45 R = Ac, R1 = ClAc, R2 = TBS
46 R = Bn, R1 = Lev, R2 = TBS
47 R = Ac, R1 = Lev, R2 = H
48 R = Ac, R1 = ClAc, R2 = H
49 R = Bn, R1 = Lev, R2 = H
HF.pyr
AcOH
41 or 42 or 43
Hexasaccharide
(c)
50
51
TMSOTf
62%
52
(d)
Cleavage to provide
1-OH donor
1st glycosylation sequence
53
54
55
56
57
2nd glycosylation sequence
Ph2SO/Tf2O for activation 1-OH
BSP/Tf2O for activation 1-SPh
58
Drug Discovery Today: Technologies

Related link

- Glycoforum: http://www.glycoforum.gr.jp/

shown that highly biologically active pentasaccharides can be obtained. Interestingly, however, the use of the non-GAG saccharides to target other biological systems has only very scarcely been reported [32].

The modular approaches that have been recently reported in literature seem to be promising for the generation of a large variety of H/HS oligomers by the combination of different (dimer) modules. However, it has also become apparent that variations in the substitution pattern of the modules used can have a profound influence on the efficiency of the assembly and can mean the difference between failure and success.

Finally, it is appropriate to compare the polymer-supported assemblies to the corresponding solution-phase syntheses. Although the former technique should be a prelude to a true solid-phase approach and offers perspectives for future automation, it suffers from the inherent low reactivity of the building blocks used. In practice, the reported polymer-supported assemblies have not been shown to be a major improvement over the corresponding solution-phase approaches. The automated and combinatorial assembly of a large library of H/HS fragments is therefore not to be expected in the near future.

In summary, several formidable syntheses of H/HS-fragments have been recorded to date, culminating in the development of a fully synthetic antithrombotic drug. However, the assembly of highly diversified H/HS-oligosaccharides still presents a great challenge and the incorporation of the full array of variations in oligosaccharides of significant length remains a daunting task even for highly skilled specialists. It is expected that the development of innovative glycosylation methodologies and novel protecting groups will continuously push the field forward and eventually can lead to a standard set of conditions for the modular assembly of a broad spectrum of H/HS-oligosaccharides.

Related articles

Yeung, B.K.S. et al. (2002) Synthesis of glycosaminoglycans. *J. Carbohydr. Chem.* 21, 799–865

Poletti, L. and Lay, L. (2003) Chemical contributions to understanding heparin activity: synthesis of related sulfated oligosaccharides. *Eur. J. Org. Chem.* 2999–3024

van Boeckel, C.A.A. and Petitou, M. (1993) The unique antithrombin III binding domain of heparin: a lead to new synthetic antithrombotics. *Angew. Chem. Int. Ed. Engl.* 32, 1671–1690

Petitou, M. and van Boeckel, C.A.A. (2004) A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? *Angew. Chem. Int. Ed.* 43, 3118–3133

Outstanding issues

- Differentiation of the amino functionalities. Although the differentiation of almost all hydroxyl functions in the heparin/heparan sulfate (H/HS) oligosaccharides can now be efficiently achieved, the differentiation of the amino groups still presents a major obstacle.
- Efficient solid-phase assembly of both non-glycosamino and glycosamino-glycans.
- Automated and combinatorial assembly techniques to access a large library of heparins and heparan sulfates.

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Figure 4. Modular approach towards heparin oligosaccharides. (a) Disaccharide modules used in the Seeberger laboratory. (b) Tetrasaccharide assembly and ensuing attempts towards the hexasaccharide via a [2 + 2 + 2] approach. (c) [3 + 3] strategy towards a highly functionalised hexasaccharide. (d) Monomeric building blocks in a sequential glycosylation sequence towards a heparin pentasaccharide (BSP, 1-benzenesulfinyl piperidine).

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